

NucleoCounter® NC-3000™ system
Application note No. 005. Rev. 1.1

Vitality assay: Analysis of the level of cellular thiols using the NucleoCounter® NC-3000™ system

Product description

The NucleoCounter® NC-3000™ system enables the user to perform automated cell counting and analyses of a broad range of eukaryotic cells.

Application

This protocol for the NucleoCounter® NC-3000™ system enables the user to detect changes in the intracellular level of (reduced) thiols. Such changes may occur in apoptotic cells or cells undergoing other pathological processes. As the intracellular reducing power available to the cell is an indicator of the overall health status, this assay provides a very easy and fast way to evaluate cell vitality.

Introduction

This application note describes a method for investigating apoptosis and cell health by determining the level of free thiols such as reduced glutathione. The tripeptide glutathione exists in two forms, a reduced state (GSH) and in an oxidized state; glutathione disulfide (GSSG). In the reduced state the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to unstable molecules such as free radicals. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form GSSG. GSH can be regenerated from GSSG by the enzyme glutathione reductase. (See **Figure 1.**)

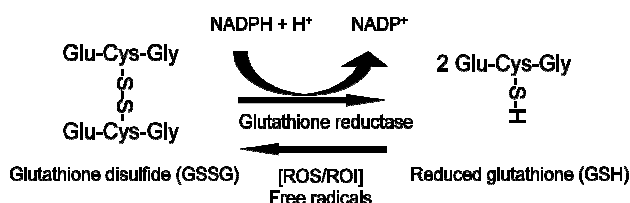


Figure 1. Glutathione redox cycle. The flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase reduces GSSG to GSH. When the cell encounters free radicals, the antioxidant GSH reduces the free radicals, thereby itself becoming oxidized to GSSG.

GSH is the most abundant low molecular weight thiol in animal cells; thus, its oxidation status largely determines the thiol-disulfide status of the cell by thiol-disulfide interchange reactions. Moreover, GSH is involved in many cellular processes including quenching of free radicals, drug detoxification, cell signaling, and cell proliferation. Alterations in the concentration of intracellular GSH have been demonstrated as a common feature of many diseases including AIDS, neurodegenerative diseases, and cancer.

A decrease in cellular GSH concentration is an early hallmark in the progression of cell death in response to different apoptotic stimuli. Studies have shown a correlation between cellular GSH depletion and the progression of apoptosis. The decrease in GSH level in connection to apoptosis seems to be attributed to two mechanisms; A) direct GSH oxidation promoted by radicals and B) export of GSH through an ATP-dependent plasma membrane transport system which is triggered by the initiation of apoptosis. When GSH is depleted, the cytosol is shifted from a reducing to an oxidizing environment, which may lead to a further depletion of GSH.

This assay provides a very easy method to quantify the amount of free thiols at the single cell level. The stain VitaBright-48 immediately reacts with thiols forming a fluorescent product. By quantifying the fluorescence it is possible to determine the level of cellular thiols, and thus determine cell health. After staining cells are loaded into either of two types of NC-Slides: the 2-chamber NC-Slide (NC-A2) or the 8-chamber NC-Slide (NC-A8).

Principle

In this application note, a method for measuring the cellular level of thiols is described. The cells to be investigated are mixed with **Solution 5**. The solution contains three different reagents: a stain staining all nucleated cells, a stain staining dead cells only and

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VitaBright-48™ which stains viable cells in an intensity-dependent manner depending on their level of thiols. A high fluorescence intensity of a particular cell indicates that the cell has a high level of thiols such as GSH. The stained cells are immediately loaded into a ChemoMetec CC1-Cassette™ or either of two types of NC-Slides: the 2-chamber NC-A2 NC-Slide or the 8-chamber NC-A8 NC-

Slide. Samples are analyzed using the NC-3000™ system. A fluorescence intensity histogram showing the distribution of thiol levels in all cells are displayed on the PC screen. By comparing histograms of treated cells to controls the fraction of cells with low vitality (e.g. apoptotic or stressed cells) can be determined.

Procedures

If the cell line to be investigated is adherent or semi-adherent, then start by getting all cells into suspension using the preferred method of your laboratory (e.g. trypsin/EDTA treatment). Although the NucleoCounter® NC-3000™ is able to count aggregated cells, the accuracy is higher for single cell suspensions.

Materials needed

- Cells to be stained
- **Solution 5** (VB-48-PI-AO)
- CC1-Cassette™ or NC-Slides (NC-A2 or NC-A8)

1. Pipette a representative cell sample from the cell suspension into a microfuge tube. Add one volume of **Solution 5** into 20 volumes of the cell suspension. E.g., if the volume of the cell suspension is 190 µL then add 10 µL **Solution 5**. Mix by pipetting.
2. Engage the NucleoCounter® NC-3000™. Turn on PC and start the NC-3000™ software.
3. Depending on the number of samples a cassette (**CC1-Cassette™**), a 2-chamber NC-Slide (**NC-A2**) or an 8-chamber NC-Slide (**NC-A8**) can be used.
 - a. **CC1-Cassette™**. Load cassette (approximately 60 µl) by immersing the tip of the cassette into the stained cell suspension and pressing the piston. Place the loaded cassette on the tray of the NucleoCounter® NC-3000™ and select the protocol "**Vitality CC1-Cassette protocol**" and press RUN.
 - b. **NC-A2**: Load 30 µl of each of the cell suspensions into the chambers of the NC-Slide. Place the loaded NC-Slide on the tray of the NucleoCounter® NC-3000™ and select the protocol "**Vitality NC-A2 protocol**" and press RUN.
 - c. **NC-A8**: Load 8 µl of each of the cell suspensions into the chambers of the NC-Slide. Place the loaded NC-Slide on the tray of the NucleoCounter® NC-3000™ and select the protocol "**Vitality NC-A8 protocol**" and press RUN.

Cellular fluorescence of cells is quantified by NC-3000™ and the VitaBright-48 intensity is displayed in a histogram. Cells with a low level of thiols (e.g. apoptotic cells) also have a low intensity score, and are thus found in the lower end of the histogram. The program has an automatic intensity cut-off for determining the fraction of cells with a low level of thiols, and based on this, the number and percentage of cells with a low vitality are presented in the result box along with the total and non-viable counts. By placing the marker, the user can divide the cell population into two (or more) subpopulations; e.g. divide the cell population into a fraction of cells with a low thiol level and another fraction with high

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thiol level, and if desired, an intermediate thiol level cell subpopulation. Typically the histogram shows two distinct populations, and in this case the marker is placed in between the two peaks (See example in **Figure 2**). The markers can be stored and retrieved in a new histogram. Thus, the position for the markers can be determined using an untreated control, and then retrieved for the treated cell sample. NB. Two histograms are shown for each sample; one histogram including non-viable cells (which has a very low level of GSH) and one histogram excluding non-viable cells.

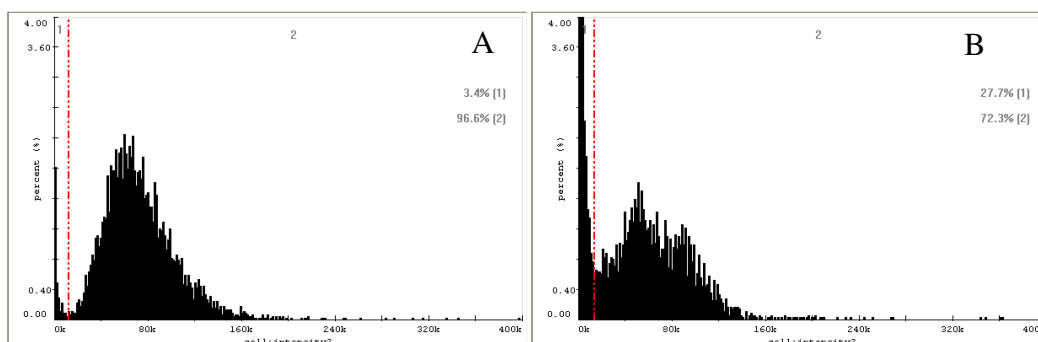


Figure 2. Fluorescence intensity histogram for untreated Jurkat cells (A) and nocodazole treated Jurkat cells (B). Nocodazole treatment causes a decrease in the level of thiols in a subpopulation of cells (cells with low fluorescence intensity).

The marker is placed in between the two populations in the (A) histogram (untreated Jurkat cells). Storing and retrieving the stored marker in histogram (B) (nocodazole treated Jurkat cells) makes quantitative comparison possible.

Note

To assure reliable results, it is recommended that the total cell concentration of the cell suspension should be in the range of $5 \cdot 10^4$ cells/mL to $1 \cdot 10^7$ cells/mL. If the concentration of cells is below $5 \cdot 10^4$ cells/mL then the cell concentration may be increased by centrifugation followed by resuspension of the pellet using growth or PBS media. The resuspended cell sample is then treated as described above.

If the total cell concentration is above $1 \cdot 10^7$ cells/mL, the cell suspension can be diluted with growth media or PBS to achieve the desired concentration. The diluted cell sample is then treated as described in the procedure.

Handling and storage

For handling and storage of ChemoMetec instruments and reagents and NC-Slides refer to the corresponding product documentation. For other reagents refer to the material data sheet from the manufacturer of the reagents and chemicals.

Warnings and precautions

For safe handling and disposal of the ChemoMetec reagents and NC-Slides refer to the corresponding product documentation and the NucleoCounter® NC-3000™ user's guide. For other reagents refer to the safety data sheet from the manufacturer of the reagents and chemicals required for this protocol. Wear suitable eye protection and protective clothes and gloves when handling biologically active materials.

Limitations

The NucleoCounter® NC-3000™ system is FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the NucleoCounter® NC-3000™ system depend on correct use of the reagents, NC-Slide and the NucleoCounter® NC-3000™ instrument and might depend on the type of cells being analysed. Refer to the NucleoCounter® NC-3000™ user's guide for instructions and limitations.

Liability disclaimer

This application note is for RESEARCH PURPOSES ONLY. It is not intended for food, drug, household, or cosmetic use. Its use must be supervised by a technically qualified individual experienced in handling potentially hazardous chemicals. The above information is correct to the best of our knowledge.

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Users should make independent decisions regarding completeness of the information based on all sources available.

ChemoMetec A/S shall not be held liable for any damage resulting from handling or contact with the above product.

Product disclaimer

ChemoMetec A/S reserves the right to introduce changes in the product to incorporate new technology. This application note is subject to change without notice.

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